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1. Neri D, et al. J Mol Biol 1995 Feb 24;246(3):367-73.
2. Portolano S, et al. Mol Immunol 1995 Oct;32(14-15):1157-69.

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MOLECULAR CLONING AND CHARACTERIZATION OF HUMAN THYROID PEROXIDASE AUTOANTIBODIES OF LAMBDA LIGHT CHAIN TYPE

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Abstract—IgG class thyroid peroxidase (TPO) autoantibodies with kappa light (L) chains predominate in serum and the genes for a large repertoire of such autoantibodies have been characterized. The present study was performed to clone and characterize TPO autoantibodies with lambda L chains which comprise ~20% of serum TPO autoantibodies. From a combinatorial IgG H/lambda L chain cDNA library in the phage display vector pComb3, 24 TPO-binding clones with lambda L chains were isolated, comprising three different heavy (H) and light (L) chain combinations. These combinations utilized two genes from the Vlambda II and IIIb families (closest germline genes *DPL*11 and *hsigg*11150) and three genes from the VH1, VH3 and VH4 families (*VH*26, 4.34 and *hv*1*L*1). The deduced amino acid sequences of these H chains were quite different from those of kappa F(ab) isolated using the same H chain library. We expressed the proteins for these three lambda F(ab), as well as for a lambda F(ab) (Humlv318 L chain/DP10-like H chain) previously isolated from another patient. The affinities for TPO of the lambda F(ab) (K_d 8 × 10^{-10} M to 10^{-7} M) were lower than those of the kappa F(ab) ($K_d \sim 10^{-10}$ M). For two lambda F(ab), both H and L chain genes were close to germline configuration, but there was no straightforward relationship between the extent of somatic mutation from germline configuration and affinity for TPO. All four lambda F(ab) bound less well to denatured TPO as to native TPO. The three F(ab) for which sufficient protein could be expressed for competition studies all recognized domain B within the immunodominant region on TPO previously identified using F(ab) with kappa L chains. Aside from these TPO-specific F(ab), only a few other human IgG class, organ-specific autoantibodies with lambda L chains have been characterized at the molecular level. Our study significantly augments the small database on this category of autoantibodies in general.

Key words: lambda V genes, TPO autoantibodies, phage display combinatorial library, autoimmunity, thyroid.

INTRODUCTION

Like most human serum antibodies, IgG-class autoantibodies to thyroid peroxidase (TPO), the hallmark of autoimmune thyroid disease, are predominantly of kappa light (L) chain type. However, lambda-type TPO autoantibodies comprise ~20% of serum TPO autoantibodies (Parkes *et al.*, 1984; Kotani *et al.*, 1986; Weetman *et al.*, 1989). The human kappa chain locus has been extensively studied and all V kappa germline genes appear to have been identified (Zachau, 1994). Furthermore, the cloning of substantial numbers of expressed antibodies of this L chain type has provided insight into kappa L chain V gene usage by human antibodies against both extraneous antigens and autoantigens (Cox *et al.*, 1994). In contrast, much less information is available on the lambda V region germline genes. Recently, a new V lambda family has been identified (Stiernholm *et al.*, 1994) and new germline genes in other families described (Williams and Winter, 1993; Irigoyen *et al.*, 1994). Despite these advances, it is likely that some V lambda genes have yet to be isolated and the information on expressed lambda chain antibodies in general remains limited.

This bias in information towards kappa, rather than lambda, antibodies also applies to human TPO autoantibodies. The genes encoding a panel of high affinity, IgG class TPO-specific autoantibodies with kappa L chains have been cloned and expressed using immunoglobulin combinatorial heavy (H) and light (L) chain cDNA libraries, generated from mRNA of thyroid-derived lym-

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phocytes from patients with autoimmune thyroid disease (Portolano *et al.*, 1991, 1992, 1993*a*,*b*; Chazenbalk *et al.*, 1993*b*; Hexham *et al.*, 1994). These kappa F(ab) define an "immunodominant region" on TPO because, (i) the region is recognized by serum TPO autoantibodies in all 42 patients with autoimmune thyroid disease so far studied; and (ii) ~80% of TPO autoantibodies in the sera of individual patients are directed at this region (Portolano *et al.*, 1992; Chazenbalk *et al.*, 1993*b*; Nishikawa *et al.*, 1994).

The goal of the present study was to expand our knowledge of the IgG class, TPO autoantibody repertoire by the cloning and molecular characterization of lambda autoantibodies. Because of the smaller proportion of lambda versus kappa L chains in serum TPO autoantibodies, combined with the problems of cloning human (rather than murine) IgG class antibodies (reviewed in Thompson, 1988; Rapoport *et al.*, 1995), this goal may be extremely difficult. Very recently, using a bacteriophage lambda vector we were only able to obtain a single lambda TPO-specific F(ab) and the very low level of protein expression precluded detailed characterization (Prummel *et al.*, 1994*b*). In the present study, we have used the potentially more powerful filamentous phage display approach (McCafferty *et al.*, 1990; Barbas *et al.*, 1991) to clone and express the genes of lambda TPO F(ab) from B cells infiltrating the thyroid, the target of the autoimmune response.

MATERIALS AND METHODS

Lambda L chain gene amplification

mRNA from thyroid tissue of a patient with autoimmune thyroid disease (WR) was reverse-transcribed (First Strand Synthesis kit, Stratacyte, La Jolla, CA). Lambda L chain cDNA was amplified by the polymerase chain reaction (PCR) (Saiki *et al.*, 1988) under the conditions previously described (Chazenbalk *et al.*, 1993*b*), using a panel of seven sense oligonucleotide primers corresponding to the 5′-end of the lambda L chain variable region and an anti-sense primer corresponding to the 3′-end of the constant region of the lambda L chain (CL). Six of the primers were the "HulambdaBACK" series (Marks *et al.*, 1991) modified by the insertion of a Sac I restriction site. The seventh primer was the VL primer of Stratacyte. For the constant region we used the CL primer of Stratacyte containing an Xba I restriction site. After restriction, the amplified lambda cDNA was gel-purified and cloned into the same restriction sites in the ImmunoZap L vector (Stratacyte), generating an L chain cDNA library of $\sim 10^6$ recombinants.

Combinatorial H/lambda L chain cDNA library construction

The WR lambda L chain cDNA parent library was combined with an ImmunoZap H chain cDNA library previously prepared from the same tissue (Chazenbalk *et al.*, 1993*b*). Reverse-transcription PCR for the H chain library involved a panel of oligonucleotide primers (Chazenbalk *et al.*, 1993*b*) designed to include all VH gene families. The combinatorial H and L chain library was constructed as previously described (Portolano *et al.*, 1991). In brief, bacteriophage DNA was purified from the amplified, individual H and L chain libraries. The H chain-containing bacteriophage arms were obtained by digestion with Hind III and Eco RI, and the lambda L chain-containing arms by digestion with Mlu I and Eco RI. Ligation of the H and L chain arms yielded a combinatorial library of 10^7 recombinants.

The WR H/lambda L cDNA in the ImmunoZap vector was transferred into the pComb3 filamentous phage vector (kindly provided by Dr R. A. Lerner, Scripps Institute) according to the strategy of Barbas *et al.* (1991). For this purpose, the combinatorial H and L chain segments in the amplified ImmunoZap library were excised with Xho I and Xba I, gel purified, ligated into the same sites in the pComb3 vector and electroporated into electrocompetent DH12S strain *E. coli* (Gibco BRL, Gaithersburg, MD) using an IBI Gene Zapper (21 μF, 2500 V, 10 msec, 400 ohms). After preparing plasmid DNA from this interim library of 10^7 recombinants, reconstitution of the pComb3 vector was completed by insertion of the 1.0 kb stuffer fragment containing the gIII fragment from pComb3 into the SacI-SpeI sites. The WR H/L chain combinatorial library in pComb3 was then electroporated into DH12S cells, as described above. XhoI-XbaI digestion of individual colonies showed that ~80% of the clones contained an insert of the correct size. Infective phagemid representative of the pComb3 library were generated by rescue with the helper phage M13KO7 (Gibco-BRL), according to the protocol of Barbas and Lerner (1991). Phagemid were precipitated with polyethylene glycol (Barbas and Lerner, 1991), resuspended in phosphate-buffered saline and stored at −20°C.

Screening of the pComb3 library

Phage from this library were screened by panning according to the procedure of Barbas and Lerner (1991). In brief, 10^{10}–10^{12} phage were applied (1 hr at 37°C) to an ELISA well coated with purified, recombinant human TPO (Kaufman *et al.*, 1991). Bound phagemid were eluted from the well in 100 μl of 0.1 M HCl, pH 2.2 (10 min at room temperature), neutralized with 6 μl 2 M Tris, pH 7.5 and then used to infect XL1 Blue *E. coli* (Stratagene)(15 min at 37°C). Aliquots of infected cells were withdrawn for titering. Culture of the remaining cells was continued overnight at 37°C in the presence of M13KO7 helper phage. The supernatant was cleared by centrifugation, the phagemids were precipitated, resuspended in phosphate-buffered saline and the panning procedure repeated three times. Antigen specificity of individual clones was tested, as previously described (Portolano *et al.*, 1993*b*), in a colony lift assay for binding to ^{125}I-TPO (~50 μCi/μg protein). Double-stranded plasmid DNA from TPO-binding clones was sequenced by the dideoxynucleotide termination method (Sanger *et al.*, 1977).

Expression of TPO-specific F(ab)

TPO-specific clones from the WR library were expressed in *E. coli* as soluble proteins using the pComb3 vector in a modification of the standard procedure (Barbas *et al.*, 1991) in which the *cpIII* gene was not removed from the pComb3 plasmid. This methodological simplification was based on preliminary experiments involving the TPO-specific kappa L chain clone TR1.21 (Portolano *et al.*, 1993*b*) in which we observed that the presence of the *cpIII* gene did not alter the affinity for TPO of the expressed F(ab) protein (K_d 1.9 $\pm$ 0.1 $\times$ 10^{-10} M, mean $\pm$ S.D., versus 3.5 + 1.1 $\times$ 10^{-10} M with and without the *cpIII* gene, respectively).

Plasmid-bearing XL1 Blue cells were incubated at 37°C in Super Broth (SB) (Barbas and Lerner, 1991) medium containing 1% glucose and ampicillin (100 μg/ml) until the optical density of the cells reached 0.5 (600 nm). After centrifugation (1000*g*) for 15 min, the cells were resuspended in SB medium without glucose and protein synthesis was induced with 1 mM isopropyl b-D-thiogalacto-pyranoside (Sigma Chemical Co., St Louis, MO) by incubation overnight at 27°C. The cells were then pelleted, frozen at −20°C, resuspended in 0.02 volumes of 10 mM Tris pH 8.0 containing 2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.1 mM phenylmethylsulfonyl fluoride (all from Sigma). Cells were disrupted by three freeze and thaw cycles, membranes pelleted by centrifugation at 30,000*g* and the supernatant containing soluble F(ab) was retained.

A TPO-specific lambda F(ab) (TR1.41), which had previously been isolated using the ImmunoZap vector (Prummel *et al.*, 1994*b*), expressed very low levels of protein and could only be studied using the filter lift assay. Internal restriction sites precluded straightforward transfer of the H and L chains into the pComb3 vector, which provides slightly greater levels of expression than ImmunoZap (Posner *et al.*, 1993). Therefore, we transferred the TR1.41 H and L chains into the Xho I and Xba I sites of pBP101 (Posner *et al.*, 1993), kindly provided by Dr B. Posner, Pennsylvania State University. Expression of TR1.41 was performed as described (Posner *et al.*, 1993) with some modifications. In brief, BL21 cells bearing the pTG119 and the pBP101 plasmids were grown at 37°C in Luria Bertani (LB) medium containing 30 μg/ml kanamycin and 10 μg/ml tetracycline (both from Sigma) until an OD of 0.8 (600 nm) was reached. Protein expression was induced by addition of 1 mM IPTG (Sigma) for 4 hr at 37°C. Cells were pelleted and processed as described above. The genes for F(ab) with kappa L chains (*SP*1.4, *WR*1.7, *TR*1.8, *TR*1.9) had previously been expressed in the pComb3 vector (Nishikawa *et al.*, 1994).

Affinity of lambda F(ab) for TPO

The binding of lambda F(ab) to ^{125}I-TPO was investigated as previously described for kappa TPO F(ab) (Portolano *et al.*, 1992). Duplicate aliquots of lambda F(ab) diluted in assay buffer (0.15 M NaCl containing 10 mM Tris–HCl pH 7.5 and 0.5% bovine serum albumin) were incubated with ^{125}I-TPO (~ 20,000 cpm) and mouse monoclonal antibody to human lambda light chains (Sigma, clone HP6054) in a total volume of 200 μl. After 1 hr at room temperature, 100 μl donkey anti-mouse SacCel (IDS, Boldon, Tyne and Wear, U.K.) was added, and the incubation continued for 30 min. After the addition of 1 ml assay buffer and vortexing, the mixture was centrifuged for 5 min at 1000*g* to sediment the immune complexes which were then counted to determine the percentage radiolabeled TPO bound. F(ab) affinities for TPO were determined by Scatchard analysis (Scatchard, 1949) of TPO binding values in the presence of increasing amounts of purified TPO after subtraction of non-specific binding (~ 3% of total counts). For the lambda F(ab) with lower affinities, Scatchard analysis could not be performed because of TPO antigen limitation. Approximate F(ab) affinities for TPO were, therefore, defined as the concentration of unlabeled TPO required for 50% inhibition of specific F(ab) binding.

Recognition by lambda F(ab) of native versus denatured TPO

These comparisons were performed using an ELISA as previously described (Portolano *et al.*, 1992), with modifications. In brief, TPO in conditioned culture medium from CHO cells (Foti *et al.*, 1990) was reduced and alkylated using dithiothreitol and iodoacetamide (Nakajima *et al.*, 1987). ELISA plates were coated with native or denatured TPO. Binding of lambda TPO-specific F(ab) was detected using murine monoclonal anti-human lambda (Sigma, clone HP6054). The signal was developed with affinity-purified anti-mouse IgG conjugated to horseradish peroxidase (Sigma) and o-phenylene diamine + H_2O_2 as the substrate and optical densities (OD) read at 492 nm. Murine monoclonal antibody no. 40.28 (kindly provided by Dr L. DeGroot, University of Chicago; diluted 1:1000) and control ascites (NS-1, diluted 1:50; Cappel, West Chester, PA) were included as positive and negative controls, respectively. For each F(ab), binding to native TPO was investigated in serial (three-fold) dilutions to obtain OD readings of 0.5–1.00.

Interaction with the immunodominant domain on TPO defined by kappa TPO-specific F(ab)

Competition by the lambda F(ab) for ^{125}I-TPO binding by the kappa F(ab), SP1.4, WR1.7, TR1.8 and TR1.9, was performed by a modification of the direct binding assay described above. In preliminary studies, we determined the dilution of each kappa F(ab) required to attain ^{125}I-TPO binding in the absence of lambda F(ab) of approximately 10–15%. For the competition studies, duplicate aliquots of increasing concentrations of lambda F(ab), as well as a control without lambda F(ab), were incubated with ^{125}I-TPO for 30 min at 25°C. The diluted kappa F(ab) were then added together with an anti-kappa murine monoclonal antibody (QE11, Recognition Sciences, Birmingham, U.K.) and the mixture incubated for an additional 30 min at 25°C prior to the addition of anti-mouse IgG (SacCel, IDS) to precipitate the kappa

F(ab), and the assay continued as described above. The percentage inhibition is expressed in relation to the values obtained in the absence of lambda F(ab), normalized to 100%.

RESULTS

Genes encoding TPO-specific F(ab) with lambda L chains

Three rounds of panning of the WR H/lambda L chain Pcomb3 library led to a progressive increase in the number of phage eluted from TPO-coated ELISA wells (Table 1). Furthermore, whereas no TPO-specific clones were detected in a sample of the original library, 90% of the clones bound ^{125}I-TPO after the final round of panning. Similarity of the nucleotide sequences of H and L chains from 24 TPO-specific clones was assessed by dideoxynucleotide sequencing of a single nucleotide ("T track"). By this method there appeared to be three patterns of H and L chain sequences. Three representative clones were chosen for complete sequencing of their variable regions.

The nucleotide sequences of the lambda L chains of these three F(ab) are most closely related to two different germline genes (Fig. 1). The WR1.102 L chain gene is ~98% homologous with the *DPL*11 germline gene (Williams and Winter, 1993), a member of the V lambda II family. In spite of being paired with different H chains (see below) the L chain genes of F(ab) WR1.107 and WR1.112 only differ from one another by five nucleotides, two of which are at the V-J junction. These L chain genes show closest homology (~98%) to *hsigg*11150 (Fang *et al.*, 1994) which belongs to the V lambda IIIb group. All three lambda L chains use *JL*2 genes (Kabat *et al.*, 1991).

Three different H chains (all of subclass IgG1) are paired with the lambda L chains of WR1.102, WR1.107 and WR1.112 (Fig. 2, Table 2). The WR1.102 H chain gene is 97% homologous to *VH*26 (VH3 family) (Matthyssens and Rabbitts, 1980). The WR1.107 H chain is most closely related (93%) to the *hv*1*L*1 germline gene (Olee *et al.*, 1992), a member of the VH1 family. Finally the WR1.112 H chain gene shares 97% homology with germline gene 4.34 (VH4 family) (van der Maarel *et al.*, 1993). The D regions of these H chains do not resemble any known D germline genes. F(ab) WR1.102 and WR1.112 use *JH*4 genes and F(ab) WR1.107 uses a *JH*6 gene (Kabat *et al.*, 1991).

It must be emphasized that the VH and VL germline genes with which the TPO-specific F(ab) genes are most homologous are provided for classification purposes. In spite of the generally high degree of homology with the indicated germline genes, particularly for the L chain genes, they may not be the actual germline genes from which the TPO-specific F(ab) are derived. Nevertheless, assuming that the closest germline genes are the actual origin of these TPO-F(ab), we have analysed the ratio of replacement/silent mutations (Table 3).

In the L chain genes of WR1.102, WR1.107 and WR1.112, the ratio of replacement versus silent mutations is higher in the complementarity determining regions (CDRs) than in the framework regions (FRs). This analysis suggests that the L chain genes of these F(ab) are generated by an antigen-driven process of somatic mutation. A similar observation was made for the H chain gene of F(ab) WR1.112, with a greater number of replacement mutations in the CDRs. However, F(ab) WR1.102 and WR1.107 have a similar rate of replacement versus silent mutations in the CDRs and FRs.

Table 1. Enrichment for TPO-binding F(ab) by panning the WR IgG H chain/lambda L chain pComb3 combinatorial library

Round of panning	Phage applied	Phage eluted	% yield
1	10^{10}	4×10^4	10^{-4}
2	10^{12}	3×10^7	10^{-3}
3	10^{12}	1×10^8	10^{-2}

Comparison with previously isolated TPO-specific F(ab) with lambda and kappa L chains

The three lambda L chains of the TPO-specific F(ab) from the WR library are quite different from the previously reported (Prummel *et al.*, 1994*b*) lambda L chain from a single TPO-specific F(ab), TR1.41 (Table 2 and Fig. 3A). TR1.41 was isolated from another patient (TR library) and its L chain is most closely related to the V lambda IIIa family germline gene *Humlv*318 (Daley *et al.*, 1992) (not *III*.1 as originally reported). The H chains of the three WR lambda F(ab) also differed from the H chain of the TR1.41 lambda F(ab) (Table 2 and Fig. 3B).

The H chain libraries used to construct the TR and WR IgG/lambda combinatorial have previously been used to construct IgG/kappa libraries (Chazenbalk *et al.*, 1993*b*). It is, therefore, of interest to compare the H chain usage of the lambda and kappa L chain F(ab) prepared from the same patient (Table 2). In the case of the WR libraries, lambda F(ab) WR1.107 and kappa F(ab) WR4.5 are both homologous to the germline gene *hv*1*L*1. However, at the amino acid level (Fig. 3B), these H chains are very different.

Turning to the TR libraries, the H chains of the TR1.41 lambda F(ab) and TR1.8 kappa F(ab) are homologous (both at the 88% level) to VH germline genes *DP*10 (Tomlinson *et al.*, 1992) and *hv*1263 (Chen *et al.*, 1989), respectively (Table 2). These two germline genes are closely related at the nucleotide and amino acid levels, 98 and 96%, respectively, and have the same canonical structure (Tomlinson *et al.*, 1992). Nevertheless, as anticipated from their low homology to germline genes *DP*10 and *hv*1263, the TR1.41 and TR1.8 VH regions are quite different from one another (Fig. 3). Further, the marked differences between the VH region of TR1.41 vs DP10, and TR1.8 vs hv1263, indicate that the V region of each H chain is highly mutated from the germline or is derived from different, as yet unreported, VH germline genes.

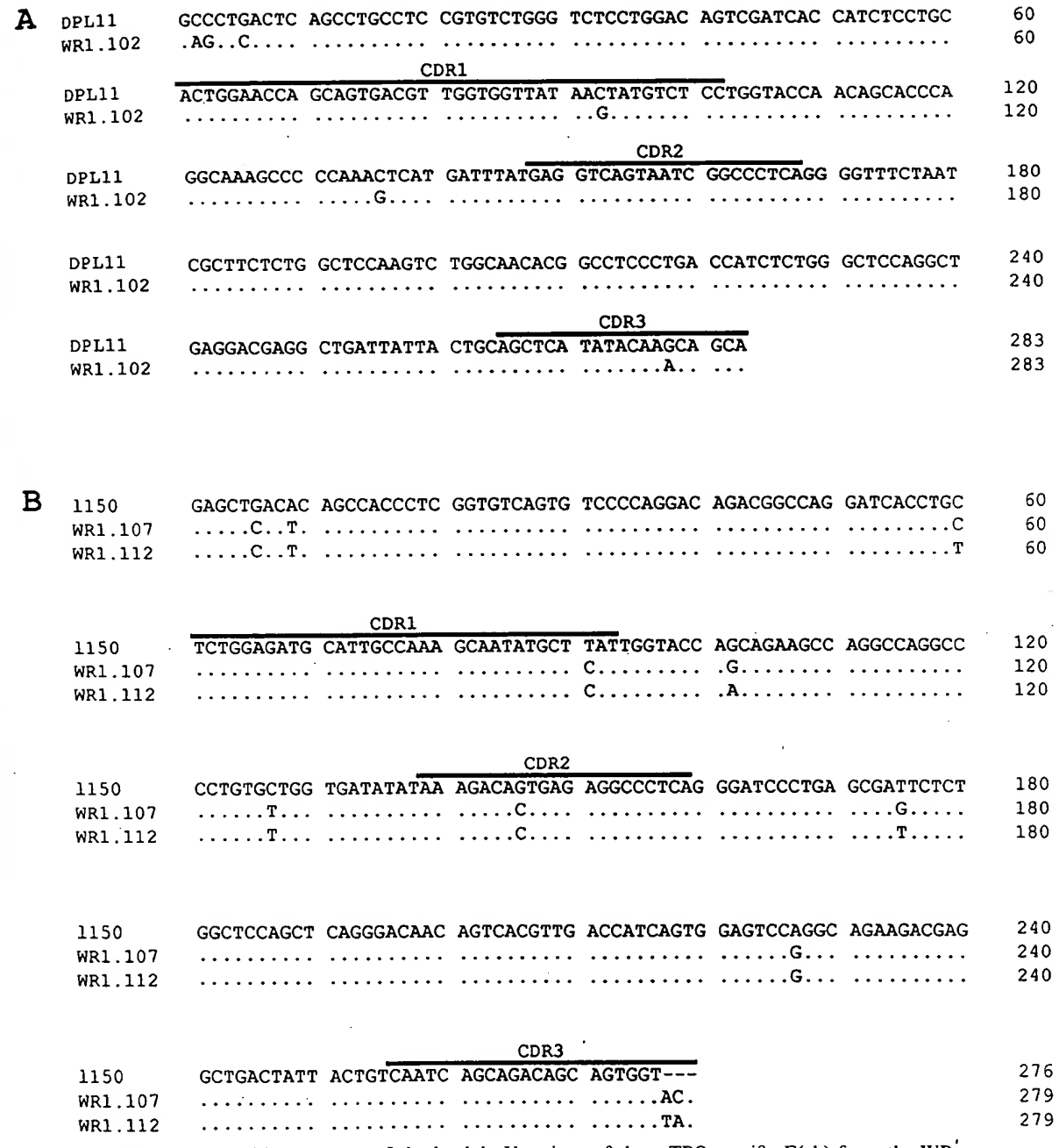

Fig. 1. Nucleotide sequences of the lambda V regions of three TPO-specific F(ab) from the WR combinatorial library. (A) F(ab) WR1.102 compared with the closest germline gene *DPL*11 (Williams and Winter, 1993). (B) WR1.107 and WR1.112 compared with the closest germline gene *hsigg*11150 (Fang *et al.*, 1994). Identities are shown by dots, base differences are indicated. Complementarity determining regions (CDRs), according to Williams and Winter (1993) for DPL11 and according to Fang *et al.* (1994) for 1150, are indicated. Nucleotide residue changes in the first nine positions of the L chains reflect the vector and the restriction site included in the oligonucleotide primer sequence.

The same caution applies to the WR1.107 and WR4.5 VH regions (see above).

Affinity of lambda F(ab) for TPO

The affinities of the lambda F(ab) for TPO was investigated by competition for binding to ^{125}I-TPO by unlabeled TPO (Fig. 4). Scatchard analysis of the data for WR1.102 and TR1.41 revealed dissociation constants (K_d) of 2×10^{-9} M and 8×10^{-10} M, respectively. The latter is consistent with the preliminary affinity data previously obtained spectrophotometrically using a confluent plaque assay (Prummel *et al.*, 1994*b*). In contrast, lambda F(ab) WR1.107 and WR1.112 had affinities too

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A
                                     CDR1                               CDR2                                                          CDR3
DPL11      ALTQPASVSG SPGQSITISC TGTSSDVGGY NYVSWYQQHP GKAPKLMIYE VSNRPSGVSN RFSGSKSGNT ASLTISGLQA EDEADYYCSS YTSS    94
WR1.102    E......... .......... .......... K......... .....V.... .......... .......... .......... .......... ..N.    94

                                CDR1                               CDR2                                                          CDR3
1150       ELTQPPSVSV SPGQTARITC SGDALPKQYA YWYQQKPGQA PVLVIYKDSE RPSGIPERFS GSSSGTTVTL TISGVQAEDE ADYYCQSADS SG-     92
WR1.107    .......... .......... .......... H......... ........T. ........V. .......... .....R.... .......... ..T     93
WR1.112    .......... .......... .......... H......... ........T. .......... .......... .....R.... .......... ..Y     93

                                  CDR1                                CDR2                                                        CDR3
Humlv318   SYVLTQPPSV SVAPGKTARI TCGGNNIGSK SVHWYQQKPG QAPVLVVYDD SDRPSGIPER FSGSNSGNTA TLTISRVEAG DEADYYCQVW DSSSD   95
TR1.41     EL.V....A. .....Q..T. S...D...T. ........A. ......ISY. TA........ .......... .......... ....F..... .R..N   95

B
                                            CDR1                   CDR2
VH26       EVQLLESGGG LVQPGGSLRL SCAASGFTFS SYAMSWVRQA PGKGLEWVSA ISGSGGSTYY GDSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCAR   98
WR1.102    Q.K....... .......... .......... N.G....... .........V ......T... A......... ........V. ...R...... ..A.....   98

                                            CDR1                  CDR2
4.34       QVQLQESGPG LVKPSQTLSL TCTVSGGSIS SGDYYWSWIR QPPGKGLEWI GYIYYSGSTY YNPSLKSRVT ISVDTSKNQF SLKLSSVTAA DTAVYYCAR   99
WR1.112    .......... .......... .......... ..S....... .N........ ...N...... ..A....... .......... .......... .........   99

                                           CDR1                    CDR2
HV1L1      QVQLVQSGAE VNKPGASVKV SCKASGDTFT GYYMHWVRQA PGQGLEWMGW INPNSGGTNY AQKFQGRVTM TRDTSISTAY MELSRLRSDD TAVYYCAR   98
WR1.107    ..K.LE..D. .RN.....RF ......Y... DS.I...... .......... ....T...RF .......... .....T.... LH....S... ........   98
WR4.5      ..K.LE.... LK......R. ...V..Y..S D.HI...... .......V.. ...KNA..R. SE........ ....A..AV. ..VTS.K... ........   98

                                            CDR1                   CDR2
DP10       QVQLV-QSGA EVKKPGSSVK VSCKASGGTF SSYAISWVRQ APGQGLEWMG GIIPIFGTAN YAQKFQGRVT ITADESTSTA YMELSSLRSE DTAVYYCAR   98
TR1.41     ..K.LE.... .......... L...L..... HN.V.T.... .......... .V..N....H ..K..L...S .......... .I.VR..T.D .........   99
TR1.8      ..K.LE.... .........R ...RT..... KNF......R .......... .F..M..ATY .......K.. ....K..NI. ..D.NT.T.. ........T   99
HV1263     .....-.... .......... .......... .......... .......... R....L.I.. .......... ....K..... .......... .........   98
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Fig. 3. Derived amino acid sequences, compared with the closest available germline genes, of the lambda V regions (A) and VH regions (B) of TPO specific lambda F(ab) WR1.102, WR1.107, WR1.112 and TR1.41. The H and L chains of lambda TPO-specific F(ab) TR1.41 have previously been reported (Prummel *et al.*, 1994*b*) (GeneBank Accession nos U09084 and U09085). Also included are the derived amino acid sequences for the H chains of kappa TPO-specific F(ab) WR4.5 (compared with WR1.107 and putative germline gene *hv1L1*) and TR1.8 (compared with TR1.41 and putative germline gene *DP10* and *hv1263*) which were previously isolated using the WR and TR H chain components of the WR and TR IgG/lambda combinatorial libraries (Chazenbalk *et al.*, 1993*b*). Identities are shown by dots, base differences are indicated. Complementarity determining regions (CDRs) are indicated.

Table 2. Comparison of the three WR lambda F(ab) with previously isolated kappa and lambda TPO-specific F(ab)

		H chain				L chain			
Light chain	Clone	VH family	Germline (%)[a]	D[b]	JH	VL family	Germline (%)[a]	JL	Sub-class
WR Libraries									
Lambda	WR1.102	3	*Vh*26 (97%)	p	4	II	*DPL*11 (98%)	2	1
	WR1.112	4	4.34 (97%)	q	4	IIIb	1150 (98%)	2	1
	WR1.107	1	*hv*1*L*1 (93%)	r	6	IIIb	1150 (98%)	2	1
Kappa	WR4.5[c]	1	*hv*1*L*1 (89%)	s	4	I	012 (93%)	2	4
	WR1.7[c]	1	*V*1-3*B* (90%)	t	4	I	012 (92%)	1	1
TR Libraries									
Lambda	TR1.41[d]	1	*DP*10 (88%)	g	3	IIIa	1*v*318 (92%)	1	1
Kappa	TR1.8[c]	1	*hv*1263 (88%)	f	3	II	*A*3 (97%)	2	1
	TR1.9[c]	1	*V*1-3*B* (95%)	c	4	I	*A*1 (97%)	4	1
	TR1.10[c]	1	*V*1-3*B* (95%)	d	4	I	012 (89%)	1	1
	TR1.3[c]	3	8-1*B* (89%)	e	4	I	012 (92%)	2	1

[a] Nucleotide homology (%) to the closest currently available germline gene.
[b] Letters are used to distinguish different D regions (Fig. 2D and Chazenbalk *et al.* (1993*b*) and Prummel *et al.* (1994*b*)).
[c] Previously reported (Chazenbalk *et al.*, 1993*b*) cloned by the bacteriophage lambda technique.
[d] Previously reported (Prummel *et al.*, 1994*b*) cloned by the bacteriophage lambda technique.

Table 3. Analysis of replacement (R) and silent (S) base changes in the H and L chain V genes of TPO-specific F(ab) WR1.102, WR1.107 and WR1.112 compared with their putative germline counterparts

	CDRs[a]			FRs[b]			
	R	S	R/S	R	S	R/S	Putative germline gene
Light chains							
WR1.102	2	0	Infinity	1	0	Infinity	*DPL*11
WR1.107	2	0	Infinity	2	2	1	1150
WR1.112	2	0	Infinity	1	3	0.3	1150
Heavy chains							
WR1.102	5	2	2.5	4	1	4	*Vh*26
WR1.107	7	0	Infinity	12	1	12	4.34
WR1.112	3	1	3	2	4	0.5	*hv*1*L*1

[a] Complementarity determining regions.
[b] Framework regions.

low for accurate determination by Scatchard analysis. Half-maximal displacements were attained at approximately 10^{-7} M unlabeled TPO, the highest concentrations of pure antigen available.

Lambda TPO F(ab) recognition of native and denatured antigen

We determined whether the lambda TPO-specific F(ab) preferentially recognized conformational epitopes

Fig. 2. Nucleotide sequences of the VH regions of three lambda TPO-specific F(ab) from the WR combinatorial library. (A) WR1.102 compared with the closest germline gene *VH*26 (Matthyssens and Rabbitts, 1980); (B) WR1.107 compared with *hv*1*L*1 (Olee *et al.*, 1992); (C) WR1.112 compared with 4.34 (van der Maarel *et al.*, 1993); (D) nucleotide and derived amino acid sequences of the D regions of lambda TPO-specific F(ab) from the WR library. Identities are shown by dots, base differences are indicated. Complementarity determining regions (CDRs) are indicated according to Kabat *et al.* (1991) for VH26 and HV111 and according to van der Maarel *et al.* (1993) for 4.34. Nucleotide residue changes in the first 16 positions of the H chains reflect the vector and the restriction site included in the oligonucleotide primer sequence.

A

		CDR1	CDR2	CDR3	
	DPL11	ALTQPASVSG SPGQSITISC	TGTSSDVGGY NYVSWYQQHP	GKAPKLMIYE VSNRPSGVSN RFSGSKSGNT ASLTISGLQA EDEADYYCSS	YTSS 94
	WR1.102	E.....	K..... .....V....		..N. 94
		CDR1	CDR2	CDR3	
	1150	ELTQPPSVSV SPGQTARITC	SGDALPKQYA YWYQQKPGQA	PVLVIYKDSE RPSGIPERFS GSSSGTTVTL TISGVQAEDE ADYYCQSADS	SG- 92
	WR1.107	....	H..... .....T. .....V.	.....R....	..T 93
	WR1.112	....	H..... .....T.	.....R....	..Y 93
		CDR1	CDR2	CDR3	
	Huml v318	SYVLTQPPSV SVAPGKTARI	TCGGNNIGSK SVHWYQQKPG	QAPVLVVYDD SDRPSGIPER FSGSNSGNTA TLTISRVEAG DEADYYCQVW	DSSSD 95
	TR1.41	EL.V....A. .....Q..T.	S...D...T. .....A.	.....ISY. TA.....	....F..... .R..N 95

B

		CDR1	CDR2		
	VH26	EVQLLESGGG LVQPGGSLRL SCAASGFTFS	SYAMSWVRQA	PGKGLEWVSA ISGSGGSTYY GDSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCAR	98
	WR1.102	Q.K.....	N.G.....	.....V ......T... A.....	.....V. ...R..... ..A..... 98
		CDR1	CDR2		
	4.34	QVQLQESGPG LVKPSQTLSL TCTVSGGSIS	SGDYYWSWIR	QPPGKGLEWI GYIYYSGSTY YNPSLKSRVT ISVDTSKNQF SLKLSSVTAA DTAVYYCAR	99
	WR1.112	....	..S.....	.N..... ...N..... ..A.....	 99
		CDR1	CDR2		
	HV1L1	QVQLVQSGAE VNKPGASVKV SCKASGDTFT	GYYMHWVRQA	PGQGLEWMGW INPNSGGTNY AQKFQGRVTM TRDTSISTAY MELSRLRSDD TAVYYCAR	98
	WR1.107	..K.LE..D. .RN.....RF	.Y... DS.I.....	....T...RF	.....T.... LH....S... 98
	WR4.5	..K.LE.... LK.....R. ...V..Y..S	D.HI.....	.....V.. ...KNA..R. SE.....	....A..AV. ..VTS.K... 98
		CDR1	CDR2		
	DP10	QVQLV-QSGA EVKKPGSSVK VSCKASGGTF	SSYAISWVRQ	APGQGLEWMG GIIPIFGTAN YAQKFQGRVT ITADESTSTA YMELSSLRSE	DTAVYYCAR 98
	TR1.41	..K.LE.... ..... L...L.....	HN.V.T....	..... .V..N....H ..K..L...S	..... .I.VR..T.D 99
	TR1.8	..K.LE.... .....R ...RT.....	KNF.....R	..... .F..M..ATY .....K..	....K..NI. ..D.NT.T.. .....T 99
	HV1263	.....-....	.....	..... R....L.I..	....K..... 98

Fig. 3. Derived amino acid sequences, compared with the closest available germline genes, of the lambda V regions (A) and VH regions (B) of TPO specific lambda F(ab) WR1.102, WR1.107, WR1.112 and TR1.41. The H and L chains of lambda TPO-specific F(ab) TR1.41 have previously been reported (Prummel *et al.*, 1994*b*) (GeneBank Accession nos U09084 and U09085). Also included are the derived amino acid sequences for the H chains of kappa TPO-specific F(ab) WR4.5 (compared with WR1.107 and putative germline gene *hv*1*L*1) and TR1.8 (compared with TR1.41 and putative germline gene *DP*10 and *hv*1263) which were previously isolated using the WR and TR H chain components of the WR and TR IgG/lambda combinatorial libraries (Chazenbalk *et al.*, 1993*b*). Identities are shown by dots, base differences are indicated. Complementarity determining regions (CDRs) are indicated.

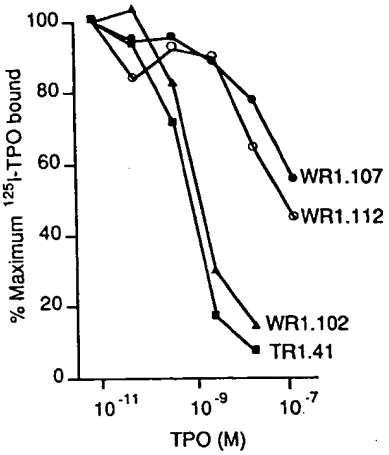

Fig. 4. Inhibition of lambda F(ab) binding to ^{125}I-TPO by increasing concentrations of unlabeled TPO. The data are expressed as % maximum ^{125}I-TPO bound. Absolute binding values in the absence of unlabeled TPO was ~10%.

(native antigen) or linear epitopes (denatured antigen). ELISA plates were coated with native TPO or with TPO previously subjected to alkylation and reduction (see Materials and Methods section). The binding of all four lambda F(ab), WR1.102, WR1.107, WR1.112 and TR1.41, was lower following TPO denaturation (Fig. 5). In contrast to the human autoantibodies, a murine monoclonal antibody (40.28), generated against denatured TPO (Portmann *et al.*, 1988) and which recognizes a linear epitope on TPO (Finke *et al.*, 1990), showed higher binding to denatured TPO.

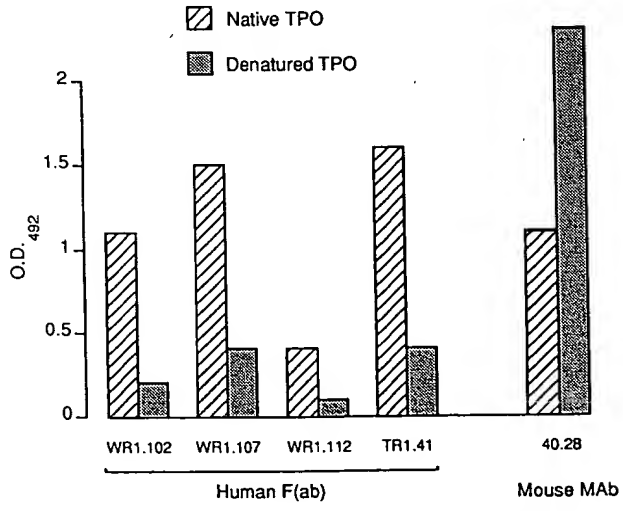

Fig. 5. Comparison of lambda TPO-specific F(ab) binding to native and denatured TPO on ELISA plates. The binding data are shown as the optical density (OD) at 492 nm after subtraction of background values (<0.05). F(ab) were diluted to give OD values with native TPO between 0.5 and 1.00. Also included are the data for a mouse monoclonal antibody (no. 40.28) which preferentially recognizes denatured TPO.

Relationship between the epitopes of the lambda F(ab) and the TPO immunodominant region

We studied the ability of increasing concentrations of lambda F(ab) to compete for the binding to ^{125}I-TPO by four kappa F(ab) previously shown to define the TPO immunodominant region (Portolano *et al.*, 1992; Chazenbalk *et al.*, 1993*b*). F(ab) WR1.102 partially inhibited the binding to TPO of kappa F(ab) TR1.8, and completely inhibited the binding to TPO of TR1.9 (Fig. 6). These two kappa F(ab) define the B domain in the TPO immunodominant region. No competition was observed with the SP1.4 and WR1.7 kappa F(ab) which define the A domain. Similar competition patterns were observed for the lambda F(ab), WR1.107 and TR1.41. With WR1.107, complete binding inhibition was obtained for both TR1.8 and TR1.9.

Insufficient lambda F(ab) WR1.112 protein could be expressed for competition studies. In our experience with both the Immunozap and the pComb3 vectors, the expression of soluble protein is highly variable among clones. For some clones, the level of functional protein produced is very low, despite the use of different cell strains and different expression protocols.

DISCUSSION

Using the WR combinatorial IgG H/lambda L chain cDNA library in the phage display vector pComb3, we isolated 24 TPO-binding clones of lambda L chain type, comprising three different H and L chain gene combinations. A comparably large number of kappa clones of similar diversity were previously isolated from WR IgG H/kappa cDNA libraries, albeit using a bacteriophage lambda vector (Chazenbalk *et al.*, 1993*b*). In contrast, we had obtained only a single TPO-specific lambda F(ab) (Prummel *et al.*, 1994*b*) but several diverse kappa F(ab) (Chazenbalk *et al.*, 1993*b*) from the thyroid tissue of a different patient (TR). In the sera of both patients, TPO autoantibodies were predominantly of the kappa L chain type. It is possible that fewer lambda F(ab) were isolated from the TR library because a bacteriophage lambda vector was used (Huse *et al.*, 1989) rather than a potentially more powerful filamentous phage vector (Barbas *et al.*, 1991; McCafferty *et al.*, 1990). The properties of the four lambda TPO-specific F(ab) are compared with representative kappa F(ab) isolated from combinatorial libraries using the same H chains in Table 4.

The three representative WR lambda TPO-F(ab) characterized in the present study are encoded by two lambda VL genes, both highly homologous (~98%) to the family II germline gene *DPL*11 (Williams and Winter, 1993) and the V lambda IIIb germline gene *hsigg*11150 (Fang *et al.*, 1994). The latter is a germline gene encoding a rheumatoid factor (Fang *et al.*, 1994). This observation is reminiscent of our previous finding that a kappa L chain gene may be used by a TPO-specific F(ab) and by systemic autoantibodies (Portolano *et al.*, 1993*b*). The L chain of the lambda F(ab) from the TR library was homologous

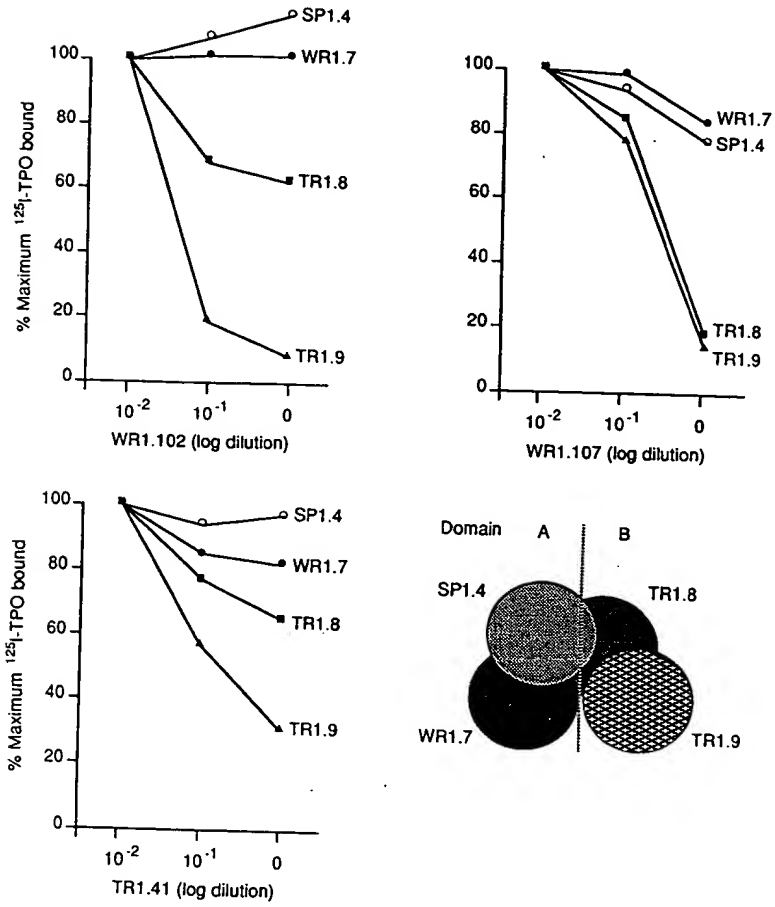

Fig. 6. Interaction of lambda TPO-specific F(ab) with the immunodominant region on TPO defined by kappa F(ab). F(ab) SP1.4, WR1.7, TR1.8 and TR1.9 define four areas within the immunodominant region on TPO (inset bottom right). Each panel shows the effect of increasing amounts of one lambda F(ab) on the binding of the four kappa F(ab) to ^{125}I-TPO. Lambda F(ab) concentrations are expressed as log dilutions of a standard for each F(ab) which bound ~10% of radiolabeled TPO. The percentage inhibition is expressed in relation to the values obtained in the absence of lambda F(ab) (~10%) normalized to 100%.

Table 4. Summary of the properties of TPO-specific F(ab) with lambda and kappa L chains from the WR and TR combinatorial libraries

F(ab)	L chain type	VH/VL genes[a]	K_d (M)	Recognition of TPO[b]		
				N > DN[c]	Domain A	Domain B
WR1.102	Lambda	*VH26/DP111*	2×10^{-9}	Yes	-	++++
TR1.141	Lambda	*DP10/lv318*	8×10^{-10}	Yes	-	++++
WR1.107	Lambda	*hv1L1/11150*	$\sim 10^{-7}$	Yes	-	++++
WR1.112	Lambda	4.34/11150	$\sim 10^{-7}$	Yes	ND[d]	ND
WR4.5	Kappa	*hv1L1/012*	3×10^{-10}	ND	++++	-
WR1.7	Kappa	*V1-3B/012*	2×10^{-10}	Yes	++++	++
TR1.3	Kappa	8-1*B*/012	5×10^{-10}	Yes	++++	++++
TR1.8	Kappa	*hv1263/A3*	3×10^{-10}	Yes	+	++++
TR1.9	Kappa	*V1-3B/A1*	2×10^{-10}	Yes	-	++++

[a] Classification of H and L chains is according to their putative germline genes based on presently available data.
[b] ++++ represents complete and - represents no overlap with the indicated domain, + or ++ indicate partial overlap. Data for kappa F(ab) from Chazenbalk *et al.* (1993*b*).
[c] N > ND; Native > denatured TPO.
[d] ND; not determined.

(92%) to a different germline gene (*Humlv*318)(Daley *et al.*, 1992) in the same V lambda IIIb family used by two of the WR lambda F(ab).

Both the WR and TR libraries were constructed with oligonucleotides designed to prime for all VL families. Indeed, using the "VL" primer (see Materials and Methods section) to construct the TR library (Prummel *et al.*, 1994*b*), we have previously isolated genes from a range of V lambda families including Ia, Ib, Ic, II, III and VII (Prummel *et al.*, 1994*a*). The WR lambda library was constructed with all six VL-specific primers of Marks *et al.* (1991) in addition to the VL primer. Nevertheless, it is of interest that all four types of TPO-specific lambda F(ab) are derived from germline genes belonging to V lambda families II and III.

Turning to the H chains of the lambda TPO-specific F(ab), the nucleotide sequences of two are close (97% homology) to their closest germline genes, *VH*26 and 4.34. Because of this high homology and because virtually all VH germline genes have now been described (Tomlinson *et al.*, 1992; Matsuda *et al.*, 1993), it seems likely that these germline genes are, indeed, the origin of F(ab) WR1.102 and WR1.112. The use of a VH26 H chain by WR1.102 is of interest because a VH26-like H chain is used by a kappa TPO-specific F(ab) isolated from a different patient (Hexham *et al.*, 1994). Further, it is the first VH3 family gene isolated using the WR H chain library. The third lambda F(ab) (WR1.107) from the WR combinatorial library is less homologous (93%) to germline gene *hv*1*L*1. The germline gene origin of the TR1.41 H chain is uncertain because of its relatively low homology (88%) to *DP*10.

A striking feature of lambda F(ab) WR1.102 and WR1.112 is that both H and L chain genes are close to germline configuration. Previously, we had isolated some TPO-specific F(ab) in which only one chain, the kappa L chain, was essentially unmutated (Portolano *et al.*, 1993*a*,*b*). Our observations parallel those previously made for murine antibodies showing that VH genes associated with V lambda genes are close to germline (Nadel *et al.*, 1993). In spite of the near germline configuration of the TPO F(ab) kappa L chains, the F(ab) bound TPO with the same high affinity ($K_d \sim 10^{-10}$ M) as TPO-specific F(ab) with more mutated L chain genes (Portolano *et al.*, 1992; Chazenbalk *et al.*, 1993*b*). It is not surprising that lambda F(ab) WR1.112, with both H and L chain genes close to germline configuration, had a lower affinity for TPO ($\sim 10^{-7}$ M). However, both H and L chain genes are also relatively close to germline configuration in WR1.102, but this F(ab) has a relatively high affinity for TPO ($K_d = 2 \times 10^{-9}$ M). There is, therefore, no straightforward relationship between the apparent lack of somatic mutation and affinity for TPO. Overall, the only generalization that can be drawn is that all four lambda F(ab), even TR1.41 ($K_d = 8 \times 10^{-10}$ M), have affinities for TPO lower than those of the previously isolated kappa F(ab) (Portolano *et al.*, 1991, 1992, 1993*a*,*b*; Chazenbalk *et al.*, 1993*b*; Hexham *et al.*, 1994).

Evidence from several laboratories suggests that H and L chains from random combinatorial libraries may not find their *in vivo* partners (Gherardi and Milstein, 1992; Burton and Barbas, 1992). On the other hand, there is evidence for selection from a combinatorial library of the H-L pairing observed *in vivo* (Caton and Koprowski, 1990). We have not observed promiscuous L chain pairing for high affinity ($\sim 10^{-10}$ M) TPO-specific F(ab) autoantibodies (Portolano *et al.*, 1993*a*; Jaume *et al.*, 1994; Costante *et al.*, 1994). However, we cannot exclude the possibility that this lack of relationship between putative somatic mutation and affinity arises because of artificial H and L chain pairing in the TPO-specific F(ab). If the lambda H and L chain pairs do indeed reflect the *in vivo* situation, the lower affinity of these F(ab) suggests that lambda TPO autoantibodies may develop later in the disease process than kappa TPO autoantibodies. This possibility is consistent with the concept that lambda L chain genes are usually rearranged after failure of kappa genes on both alleles to rearrange in a productive manner (Hieter *et al.*, 1981).

TPO-specific kappa F(ab), like most serum TPO autoantibodies, interact preferentially with conformationally intact antigens (Chazenbalk *et al.*, 1993*a*). However, a minority of serum TPO autoantibodies do recognize denatured TPO and two linear TPO B cell epitopes have been described (reviewed in McLachlan and Rapoport, 1992). As might be expected, serum TPO autoantibodies in patient WR preferentially recognize native TPO and interact to a lesser extent with denatured TPO. Further, the WR pComb3 library was screened against recombinant TPO on an ELISA well, most of which is in native condition. However, from studies using mouse monoclonal antibodies to denatured antigen, it is clear that a small proportion of this TPO is denatured (Portolano *et al.*, 1992; Chazenbalk *et al.*, 1993*a*). For these reasons, it was of interest to determine whether the lambda TPO-specific F(ab) preferentially recognized native or denatured TPO. All four lambda F(ab), including two with lower affinities for TPO, bound better to native than to denatured TPO. In addition, we determined whether or not the epitopes for the lambda TPO-specific F(ab) overlapped with the immunodominant region defined by the F(ab). The three F(ab) for which sufficient protein could be expressed, all recognized domain B within this region.

In conclusion, the present data represent a major expansion of available information on TPO-specific lambda F(ab) from B cells infiltrating the thyroid in autoimmune thyroid disease. The proteins expressed by these F(ab), together with another lambda F(ab) previously isolated from a different patient, have lower affinities for TPO ($K_d \sim 10^{-7}$ M to $\sim 10^{-9}$ M) compared to the TPO-specific F(ab) with kappa L chains ($\sim 10^{-10}$ M). Of interest is that both H and L chain genes encoding two of these F(ab) are close to germline configuration. Apart from these TPO-specific F(ab), only a few other human IgG class, organ-specific autoantibodies with lambda L chains have been characterized at the molecular level (reviewed in Rapoport *et al.* (1995)). Our study significantly augments the small database on this category of autoantibodies in general.

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